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Összefoglaló közlemények / Reviews

Recent advances in the application of liquid biopsy in gastrointestinal malignancies

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Liquid biopsy is an emerging diagnostic tool in medicine. That means non-invasive sampling and analysis of whole blood, serum, saliva and urine samples for cancer-associated particles (e.g. circulating tumor cells, circulating cell-free nucleotides, exosomes, cancer associated proteins, etc.). Liquid biopsy could develop to be a crucial step in the diagnosis and management of gastrointestinal tumors. Compared to currently used tissue based (core biopsy, fine-needle aspiration biopsy, surgical resection) diagnostic methods it might deliver comparable information regarding molecular and genetic profiling of tumors. Further, it might provide earlier diagnosis and better reflect tumor heterogeneity. Used together with other diagnostic modalities (e.g. imaging studies, currently used tumor markers) liquid biopsy could provide useful information for precision medicine and personalized therapy.
KEYWORDS: liquid biopsy; gastrointestinal; liver; cancer, blood

A likvid biopszia új felhasználási lehetőségei gasztrointesztinális daganatokban

A likvid biopszia egy feltörekvő módszer az orvosi diagnosztikában. Ez noninvazív módon nyert vér-, nyál- és vizeletminták analízisét jelenti, különböző tumorasszociált partikulumok (pl. keringő tumorsejtek, keringő nukleotidok, exoszómák, tumorasszociált fehérjék) kimutatására. A likvid biopszia a jövőben lényeges lépéssé válhat az emésztőtraktus daganatos betegségeinek diagnosztikájában és kezelésében. A módszer az eddig használt szövetalapú mintavételi technikákhoz (sebészi reszekció, core biopszia, vékonytű-aspiráció biopszia) képest potenciálisan ugyanannyi vagy több információt tud nyújthat a tumorok molekuláris és genetikai hátteréről. Ezen túlmenően koraibb diagnózist eredményező és a tumorheterogenitást jobban tükröző módszer lehet. Egyéb diagnosztikus eljárásokkal (pl. képalkotási módszerek, jelenleg is használt tumormarkerek), a likvid biopszia a precíziós orvoslás és a személyre szabott terápia számára szolgálhat hasznos információkkal.
KULCSSZAVAK: likvid biopszia, gasztrointesztinális, máj, rák, vér, tumor

Introduction

Liquid biopsy in medical diagnostics is based on the analysis of body fluid samples (e.g. whole blood, serum, urine and saliva, etc.) with the purpose of screening, diagnosing and monitoring diseases. Major targets are malignant tumors that are difficult to detect with routinely applied clinical methods, such as imaging modalities and solid biopsy methods (fine-needle aspiration biopsy – FNAB – core biopsy). The primary advantage of this non-invasive method when compared with imaging methods is the potential ability to detect malignant diseases in their early stages, which might not always be possible to accomplish due to the limited resolution of imaging techniques.

Samples taken via conventional biopsies are suitable for cytological, histological or molecular analysis and might serve to establish early diagnosis, identify targets for

therapy and determine prognosis or prediction. However, there is the possibility that even numerous biopsy specimens do not represent the complexity of the tumor due to tumor heterogeneity (1).
Furthermore, early metastases might remain undetectable with routine clinical methods after resection of the primary tumor. In addition, there are anatomical situations where conventional biopsies cannot be performed due to technical difficulties, while blood, saliva and urine samples are readily available under most circumstances. Therefore, body fluids could serve as non-invasive sampling for sensitive detection methods. Various circulating particles, such as circulating tumor cells (CTCs), circulating cell-free nucleotides (cfNAs), exosomes and cancer-associated proteins could be investigated.
Widespread use and further evaluation of the liquid biopsy technique could lead to breakthroughs in cancer diagnostics and screening. Subsequently, early detection, molecular and genetic profiling of tumors could guide chemotherapy and targeted therapy, as well as monitoring patients for relapses.
The aim of this article is to review recent advances and methods in the application of liquid biopsies in the research and clinical setting, mainly from the perspective of malignancies of the gastrointestinal tract.

Sampling of liquid biopsy

Blood/serum

Blood samples are the most common form of liquid biopsy by far, due to the ubiquitous nature of this sampling method. The number of studies evaluating potential blood biomarkers for solid tumors is ever-growing and major advancements have been made towards development of universal tumor blood biomarkers.
CancerSEEK is a multicomponent protein- and circulating tumor DNA (ctDNA)-based combined blood test. Based on the detection of somatic mutations in the eight most common solid tumors (lung, colorectum, breast, ovary, liver, pancreas, esophagus and stomach) and elevated plasma levels of eight proteins (CA-125, CEA, CA19-9, PRL, HGF, OPN, MPO and TIMP-1), CancerSEEK has a sensitivity of over 95% in detecting ovarian and liver tumors, approximately 70% for stomach, pancreatic and esophageal cancer, and nearly 60% for colorectal (CRC) and lung cancer (LC). Sensitivity for breast cancer was significantly lower. This study also concluded that mutations detected through the analysis of cancer tissue biopsies were also detectable in the blood samples in approximately 90% of the cases examined (2).
Methylation patterns, the so-called Methylscape, of genomic DNA, are drastically altered in tumor cells when compared with non-cancerous cells. Epigenetic reprogramming results in significantly higher quantities of 5-methylcytosine in DNA derived from cancer cells. This changes the physical properties of purified genomic DNA (gDNA), leading to significant differences in the solvation properties of gDNA when compared with gDNA from non-cancerous cells. Results showed that cell free DNA (cfDNA)-derived gDNA samples of cancer cells had higher affinity towards bare gold surfaces in comparison to that of non-cancerous cells. Sample pre-processing or amplification was not necessary in this method. Analysis of Methylscape could universalize detection of cancer-associated nucleotides in samples irrespective of origin (e.g. plasma, tissue) and cancer location (3).

Saliva

Analysis of saliva samples has the potential to become a major tool of cancer diagnostics due to its technical simplicity and feasibility (4).
Detection of microRNA-21 (miR-21) was found as a plasma and saliva marker of colorectal cancer (CRC). Both plasma and saliva levels of miR-21 were elevated in saliva samples taken from patients having colorectal cancer at various stages, producing a sensitivity and specificity of 97% and 91%, respectively, while only 65% and 85% in plasma samples. Thus, in this study, saliva-based liquid biopsies showed higher sensitivity and specificity compared to plasma samples (5).

Urine

There are reported investigations for the use of urine samples to diagnose colorectal cancer.
Song et al. used urine samples of 150 metastatic colorectal cancer (mCRC) patients to analyze KRAS mutations of ctDNA. Concordance of 90% was found between the results from surgical resection specimens and urine ctDNA samples regarding KRAS mutational status (6). A high concordance rate between the results of invasive tissue sampling and non-invasive methods might lead to the reassessment of diagnostic and surveillance protocols.

Ascites

Ascites could serve also for liquid biopsy. Tumor-infiltrating lymphocyte (TIL) profile of ascites fluid samples of patients with high-stage gastrointestinal (colorectal, gastric and pancreatic) tumors was studied by *Nakano et al.* Results of this study showed that ascites fluid TILs exhibiting PD-1 and/or TIM-3 positivity were indicative of poor prognosis (7).

Targets of liquid biopsy

Cell-free DNA and RNA (cfDNA, cfRNA)
Circulating cell-free DNA (cfDNA) is predominantly derived from circulating leukocytes in blood samples, with a significantly smaller fraction originating from circulating tumor cells (8, 9).
Analysis of cell-free DNA has been used in various fields, including prenatal screening, detection of infectious diseases such as EBV (HHV-8), hematological malignancies and for early screening for solid tumors as well (10, 11).
Analysis of circulating DNA might provide more precise information on heterogenous mutation profile of the tumor cells than molecular analysis of conventional biopsy. This feature could be useful to monitor and guide personalized, targeted therapy (12).

Circulating tumor cells (CTCs)

CTCs are tumor cells detectable in blood samples which are associated with the presence of solid tumors. CTCs might exhibit similar features of the primary tumor or might reflect the heterogeneity of molecular and genetic profile of the primary malignancy. Analysis of these potential similarities and differences is one of the most contested topics of non-invasive tumor diagnostics. The technique might also allow early detection of tumor invasion (13). However, a major limitation to the diagnostic and prognostic utility of circulating tumor cells of epithelial origin is the possible epithelial-mesenchymal transition (EMT) of CTCs, which leads to loss of surface EpCAM expression (14).

Circulating microRNAs (miRs)

microRNAs (miRs) act as important factors in the negative regulation of gene expression. Several studies detail their role as oncogenic and tumor suppressor miRs as well as biomarkers and therapeutic targets. Further, they might bear predictive information on survival. They are present in tumor cells and also in body fluids as circulating tumor-associated miRNAs (15).

Exosomes, extracellular vesicles

Exosomes are circulating vesicles found in numerous body fluids, including blood, saliva and urine (16). Exosomes might contain miRs, long non-coding RNAs, piwi-interacting RNAs, transfer RNAs and small nuclear RNAs (17).

Proteins

Carcinoembryonic antigen (CEA) and Alpha-Fetoprorein (AFP) have been used as tumor markers for monitoring of disease progression and therapeutic efficacy in colorectal cancer and primary cancers of the liver, respectively (18, 19). Cancer Antigen 19-9 (CA-19-9) has been used as a tumor marker primarily for pancreatic malignancies. However, increased plasma CA19-9 has also been associated with colorectal malignancies and pancreatitis (20).

Tumor markers have been widely used for monitoring progression and therapeutic response. However, neither their sensitivity nor specificity are high enough for them to be used as diagnostic tools. Therefore, future protein biomarkers of cancers must demonstrate high sensitivity and specificity.

Associations with microbiome changes

There is established evidence of associations between the presence of certain microbial agents detected in body fluid (e.g. blood, joint aspirate etc.) samples and asymptomatic malignant tumors. Due to these associations, bacterial culture and sensitivity tests in the workup of infective endocarditis could also be considered "preemptive" liquid biopsies (21, 22).

One prominent example is *Streptococcus gallolyticus* (group *G Streptococcus*, formerly called, *Streptococcus bovis*) bacteremia and endocarditis, which has been shown to be associated with asymptomatic colorectal, pancreatic and biliary neoplasms. Inflammatory conditions of prosthetic and non-prosthetic joints with aspirates positive for *S. gallolyticus* have also been described as warning signs of underlying malignancies (22).

There are reports of other microbial agents, such as *Streptococcus* viridans and *Enterococcus* faecalis being among the incidental first indicators of with hidden malignant tumors, primarily colorectal carcinomas (23, 24).

Liquid biopsy of gastrointestinal tumors

Hepatobiliary tumors

Hepatocellular cancer (HCC)

Lee et al. elaborated the critical importance of genes FGF19 and MET in the targeted therapy of hepatocellular carcinomas (HCCs). Detailed molecular pathological analysis is essential for establishing the appropriate therapeutic regiment, which necessitates the acquisition of several HCC samples. Due to tumor heterogeneity, even several conventional core biopsy samples may not provide representative information about the entirety of the tumor. On the other hand, it was observed that liquid biopsy samples could better represent the mutational status of the HCCs examined by detecting mutations of the FGF19 and MET genes in cases where mutations could not be discovered by analysis of solid tissue biopsies alone (25).

miR-224 is an oncogenic microRNA with highly elevated levels in HCCs that has been described in numerous studies as a prognostic marker independent of hepatic function. *Okajima et al.* described that miR-224 levels in plasma and HCC tissue samples were significantly correlated (26). Elevated expression of miR-224 in HCC cells collected by FNAB before sorafenib treatment is associated with higher overall survival rates (15). Detection of miR-224 in plasma samples may become a method to screen for HCCs, while also providing valuable information prior to surgery regarding possible disease outcome.

Downregulation of miR-195 expression was described as a phenomenon associated with the presence of HCC. Blood samples of 120 patients with HCC, 64 patients with hepatitis and 118 healthy controls were analyzed and miR-195 levels were assessed using qRT-PCR, with relative expression of miR-196 normalized to that of miR-16. Blood levels of miR-195 were significantly lower in samples taken from HCC patients when compared with samples of patients with hepatitis without cancer, as well as and healthy controls. Samples of HCCs with concurrent hepatitis also exhibited lower miR-195 levels compared to that of hepatitis patients without cancer. HCCs with low miR-195 were associated with higher TNM stage, particularly the presence of lymph node metastases, in comparison to high miR-195 cases. These results could also suggest a potential role for miR-195 as an agent in the targeted therapy of HCCs (27).

Low expression of beta-lactamases (LACTB) in HCCs is associated with tumor progression and poor prognosis. Based on two cohorts of 396 and 84 HCC tissue samples and the associated clinicopathological data, low expression of LACTB showed correlations with tumor sizes >5 cm and higher TNM stage. Risk of portal vein thrombosis was almost twice as much in cases with low expression of

ACTB when compared with tumors of high ACTB expression. Expression of ACTB was described as an independent prognostic factor in HCC patients, with low ACTB expression. HCCs being associated with poor patient survival (28).
Serum laminin γ2 monomer (Ln-γ2), a component of Laminin-332 (Ln-332) trimer, is a novel marker for HCC. Earlier studies described the presence of monomeric Ln-γ2 in high quantities in aggressive variants of HCCs. Serum levels of monomeric Ln-γ2 were analyzed in 57 patients with HCC, 24 patients with chronic liver disease and 52 healthy controls using chemiluminescent immunoassays (CLIA). Monomeric Ln-γ2 was highly elevated in samples of HCC patients compared to those with chronic liver disease and healthy controls. Serum AFP and desgamma-carboxyprothrombin (DCP) levels were also measured. Serum monomeric Ln-γ2 was described as a biomarker superior to AFP in the differential diagnosis of HCCs versus non-cancerous chronic liver disease. A combination of DCP and Ln-γ2 was found to have a higher chance of detecting HCCs in comparison to the combination of AFP and DCP, as well as any of the three markers separately (29).
Platelet-derived Growth Factor BB (PDGF-BB) is a cytokine of critical importance in the process of carcinogenesis. Pre- and postoperative serum PDGF-BB levels were measured in patients who underwent curative resection of HCCs and monitored for HCC recurrence. Patients who developed recurrent HCCs had significantly lower serum PDGF-BB levels both preoperatively and postoperatively compared to HCCs with high serum PDGF-BB. These findings could point to perioperative serum PDGF-BB levels as early predictors of HCC recurrence (30).
A recent study scrutinized the use of CTCs in differentiating HCC from non-cancerous diseases of the liver. CTCs were assigned into 3 categories based on surface marker expression profiles determined via the use of CTC assays: epithelial (EpCAM, CK8, CK18, CK19), mesenchymal (Vimentin, Twist) and mixed, with total CTC count also used as a parameter. Results indicated that total CTC counts were superior to the use of serum AFP levels in confirming or excluding the presence of HCC. Combined use of total CTC count and AFP provided better results than either methods alone, suggesting that CTC analysis could potentially become a modality of HCC diagnosis equal or superior to routine AFP measurements (31).
Examining CTCs as potential indicators of postoperative survival in HBV-associated HCCs found significant correlations between preoperative CTC count and Edmondson stage. Lower postoperative CTC count and perioperative changes in CTC count were associated with higher progression-free survival (PFS), but no correlation was found with OFS. The most sensitive predictor of PFS was determined being the perioperative changes in CTC counts. However, there were no associations between CTC count and any clinicopathological features (32).

Cholangiocarcinoma (CCA)

The use of fluorescence-activated cell scanning (FACS) for the detection of tumor-associated microparticles (taMPs) was elaborated in the differentiation of hepatobiliary tumors (HCC, CCA) from taMPs detectable in colorectal and lung cancers, as well as observing differences in the taMP profiles of cancerous and non-cancerous diseases of the liver, such as liver cirrhosis. Results showed that both major categories of hepatobiliary tumors (HCC and CCA) were associated with elevated serum levels of AnnexinV+ EpCAM+ CD147+ taMPs.
A marked decrease in postoperative serum levels of AnnexinV+ EpCAM+ ASGPR1+ taMPs was also observed in patients who had undergone curative surgical resection of their liver malignancies. No such correlation was noted between liver cirrhosis without malignancy and AnnexinV+ EpCM+ ASGPR1+ taMPs (33).
Postoperative monitoring of residual tumor burden or recurrence could become a major task for liquid biopsies. Minute quantities of detectable particles associated with the presence of cancer could serve as indicators of incomplete resection and/or relapse, potentially providing crucial information earlier than imaging studies and currently used tumor markers.
Peptidase Inhibitor 15 (PI15) was first described in human glioblastoma and neuroblastoma cells (34). Studies also discovered that PI15 is absent from non-tumorous human liver tissue while being abundant in CCA and present, to a lesser degree, in HCC, patients with HBV and benign liver disease. These results prompted another study, in which blood PI15 levels were described as a specific marker of CCA. Plasma PI15 levels were measured in 61 CCA patients, 72 patients with HCC, 45 patients with chronic HBV infection and 28 cases of benign liver disease and quantified using ELISA. Concentration of PI15 was significantly higher in patients with HBV-negative, but not HBV-positive CCA when compared with other sample groups, which suggested a role for PI15 as a potential diagnostic marker specific for CCA.
Simultaneous use of CA19-9 and PI15 was also investigated in this study, with the results showing increased accuracy in diagnosing CCA and detecting postoperative residual tumor mass, further reinforcing the reliability of PI-15 as a potential diagnostic tool (35).

Colorectal cancer

It is established that pre- and post-treatment levels of CTCs in metastatic colorectal cancer are predictive of patient survival; in a 2008 publication by *Cohen et al*., higher detectable CTC levels prior to and following treatment indicated poor progression-free and overall survival when compared with patients with lower CTC levels in a study involving 430 patients. Differences between pre- and post-treatment CTC levels were also found to be of notable significance, with a "conversion" from a high baseline CTC level to a lower one following treatment being a predictor of better outcomes than that of those retaining high CTC posttreatment (36).
A Meta-analysis by *Rahbari N, et al.* concluded that the presence of CTCs in patients with colorectal malignancies is indicative of poor prognosis (37).
Hinz S, et al. observed that higher CK20 expression of CTCs is indicative of poor clinical outcome in CRC patients (38).
Hendricks A, et al. further described the successful use

of CK20 and EGFR detection in peripheral mononuclear blood cells (PMBCs) using RT-qPCR in preoperative blood samples of colorectal cancer patients (39).
Extracellular miR-21 levels were studied in patients with colorectal cancer. Results showed that elevated exosomal levels of hsa-miR-21 in colorectal cancer patients were indicative of higher TNM status, liver metastases and poor prognosis when compared with patients exhibiting low levels of exosomal miR-21 (40).
Comparative study of cell-free DNA (ctDNA) and circulating tumor cell (CTC) detection in stage IV colorectal cancer observed that while cfDNA could be isolated from the samples of every patient included in the study, CTCs could only be detected in about one out of every three cases. Liquid biopsy findings were compared with those from tissue samples, finding a concordance of over 80% between ctDNA and tissue samples regarding RAS, BRAF and ERBB2 alterations (41).
This suggests that cell-free tumor DNA (cfDNA) might be present in higher, more reliably detectable quantities in blood samples of CRC patients than CTCs.
RAS mutation testing in liquid biopsy samples of patients with metastatic colorectal adenocarcinomas was found to have a concordance rate of 78.3% compared with tissue sample analysis (42).
Recent study of cfDNA sequencing in patients with metastatic CRCs (mCRC) found that mutations of TP53, KRAS and APC genes detected in plasma samples are associated with metastasis formation and correlate with tumor markers (CEA, CA-19-9) and tumor size. This study used an amplicon-based NGS platform to look for mutations of 14 genes covering a broad spectrum of possible genetic alterations in CRCs. Multi-gene panels like this could potentially be used for monitoring response to chemotherapy in mCRC patients (43).
A study of exosomal ECM1 expression in blood samples taken from peripheral blood and tumor-draining mesenteric veins showed that exosomal ECM1 levels are elevated in the mesenteric vein blood of CRC patients with relapses when compared with relapse-free individuals and healthy controls. However, obtaining samples from tumor-draining mesenterial veins is possible only during a surgery, an invasive procedure. Furthermore, levels of exosomal ECM1 in the mesenterial blood samples differed from those observed in peripheral blood: exosomal ECM1 expression in the peripheral blood of relapse-free patients was higher in comparison to both relapsed patients and controls (44).

Conclusions

Liquid biopsy is a collection of emerging non-invasive diagnostic methods with the potential to become a widely used tool in the management of cancer patients including screening, diagnosis and follow-up. However, further methodological elaboration is needed, which must establish a library of verified biomarkers for each type of cancer.
Additionally, as it was demonstrated by the articles cited in this review, analysis of multiple circulating particles of more than just one type (e.g. proteins, cfRNA, cfDNA, exosomes etc.) is more strongly associated with correct diagnosis.
Thus, the liquid biopsies of the future will likely include panels that can quantify the expression of several biomarkers. Used in tandem with already established modalities of diagnosis and surveillance (e.g. imaging studies, tumor markers etc.), liquid biopsy could emerge as a crucial method providing information regarding the clinicopathology of tumors using non-invasive methods.

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